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Investigation of Ultrasonic Parameters – Effect of High Frequency Ultrasound on Disruption of Cell Density of *A.niger* and *P.chrysogenum*

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Abstract: ***The high ultrasound frequencies namely 2 MHzand 10MHz frequencies were selected for disruption of cell density of A.niger based on Rayleigh-plesset's bubble activation theory. The A.niger suspensions of 10 ml each inoculated to cell density of 10^7 per ml were exposed to ultrasound of out power of 60 watts/ml for times of 1 min step up to 10 min. A sonotrode of 12mm diameter was used throughout the study. The sonication vessel kept inside the water bath which was maintained at constant temperature of 300 K by using automatic temperature control bath. The ultrasonic velocity and attenuation coefficients were measured by using Pulse Echo Overlap (PEO) technique. Jacobson's free length theory (FLT) was applied to calculate thermo dynamical parameters like compressibility, free length and acoustical impedance. The disruption of A.niger cells as a function of ultrasound exposure was ascertained with viable cell density that was measured by using Hemocytometer.It was noticed that ultrasonic velocity and attenuation coefficient linearly decreased with ultrasound exposure time. The onset of transient cavitation with increased ultrasound exposure initiates cell disruption that causes to linear decrease of ultrasonic velocity and attenuation coefficient. The initiation of disruption process caused to decrease of free length and compressibility of suspension could be the primary reason to decreased ultrasonic velocity and attenuation coefficient. The results of ultrasonic velocity and attenuation coefficient of A.nigerand P.chrysogenum were found in the right order of magnitude and confirmed with literature values of A Zips and U Fast. It was noticed that the rate of variation of ultrasonic parameters on disruption of cell in suspensions occurred early at 2 MHz than 10MHz for A.niger and the reverse trend was observed for P.chrysogenum. It was concluded that the fundamental interactions between the ultrasound and cell in suspensions and their sizes were responsible for variation of ultrasonic parameters.***

Keywords: A.niger, P.chrysogenum, Ultrasound, Pulse Echo Overlap (PEO) method, Ultrasonic velocity and Attenuation coefficient

1. Introduction

In the recent years, the measurement of ultrasonic velocity and attenuation coefficient has been adequately employed in understanding biological effects induced by ultrasound in macromolecules, microorganisms, tissues, organs and bones etc. Beside widespread well established studies of ultrasonic velocity and attenuation coefficient of biological materials in basic research, more recently the adiabatic compressibility and intermolecular free length has also been considered as an indicator with high potential in the advanced medical diagnoses and as a process control parameter in the food industry [1]-[3].

The lot of research has been carried out by researchers dealt with disruption and inactivation of microbes depending upon the power, pressure and frequency applied through a number of physical, mechanical and chemical effects arising from acoustic cavitations [4]-[6].

Resistance of different species to ultrasound differed widely which was because specific effect of ultrasound on the cell wall and differences in the cell wall structures among species [7]. Bacterial spores were much more resistant than vegetative ones and fungi were more resistant in general than vegetative bacteria [8]. Since molds and yeasts were in general more resistant to high intensity ultrasound [9] and not enough information about mold spores was available.

The velocity of an ultrasound wave through a medium varies with the physical properties of the medium. Liquids exhibit ultrasound velocities intermediate between those in gases and solids. With the notable exceptions of lung and bone, biologic tissues yield velocities roughly similar to the velocity of ultrasound in liquids. In different media, changes in velocity are reflected in changes in wavelength of the ultrasound waves, with the frequency remaining relatively constant [10].

The velocity of sound was determined by the density (ρ) and compressibility (K) of the medium. Density was the amount of material in a given volume, and compressibility was a measure of how much a substance could be compacted for a given pressure. The denser and the more compressible, the slower the sound waves would travel. Water was much denser than air, but since it is nearly incompressible the speed of sound was about four times faster in water than in air. The speed of sound in a medium can be determined by the equation

$$v = (K\rho)^{-½}$$

Where v is the speed of sound, K is the compressibility, and ρ (rho) is the density.

The various forms of interaction between ultrasonic waves and particles was necessary to understand how ultrasound can be used to characterize microbes [11], [12]. The four most important types of interaction between an ultrasonic wave and a colloidal dispersion of microbes were intrinsic absorption, visco-inertial dissipation losses, thermal dissipation losses and scattering losses.

Each of the individual component phases in a colloidal suspension absorbs ultrasound as a result of classical(viscous and thermal) and relaxation loss mechanisms. These losses were determined by the composition of the suspension, rather than by its microstructure.

The pulsation and oscillation of a particle in the presence of an ultrasonic wave causes the generation of secondary ultrasonic waves by the particle. Thus some of the ultrasonic energy associated with the incident wave was redirected into different directions, and an increase in the attenuation coefficient may be detected. Scattering losses were usually negligible in the long wavelength limit, but become dominant when the particle size was of the same order of magnitude as the ultrasonic wavelength. Scattering losses depend on the size of the microbes in a colloidal suspension.

To a first approximation, the overall attenuation coefficient of a colloidal suspension of microbes can be considered to be the sum of these various contributions (although in reality some of these mechanisms were coupled to each another).In most suspensions, one or two of the above mechanisms usually dominate the overall attenuation in a particular frequency range.

A.niger and P.chrysogenum were chosen as representative species. In terms of ultrasonic measurements more research is necessary to understand their use to estimate cell density and disruptive applications in food industry, especially for fungi. The purpose of this research was aimed to measure density, ultrasonic velocity, attenuation coefficient, and evaluates compressibility, free length and acoustical impedance as a function of cell density and sonication time.

2. Materials and Methods

2.1 Sample collection

The samples of rice seed, rice bran, wheat bran and cheese were collected in sterilized screw capped bottles and transported to the Advanced Microbiology Laboratory, University College of Technology. The samples were stored under refrigeration conditions for conducting different experiments.

2.2 Sample preparation

From each sample water suspensions were made by dipping the samples in pre-sterilized distilled water and these suspensions were used for isolation.

2.3 Media preparation

The potato dextrose agar (PDA) and potato dextrose broth (PDB), used for fungal growth were prepared according to the methods recommended by Harrigan (1998) [13]. The pH of media was adjusted by using 0.1N NaOH and 0.1N HCl.

2.3.1 Potato dextrose broth

The diced potatoes were boiled in 1 L of distilled water for 1 hour and then filtered through muslin cloth. The volume of filtrate was made upto 1000 mL and then glucose was added. The medium was sterilized by autoclaving.

Composition of potato dextrose broth	
Ingredients	Quantity
Potatoes peeled and diced into small pieces	200g
Glucose	20g
Distilled water	1000 mL

2.3.2 Potato dextrose agar

The potato dextrose agar (PDA) was prepared by adding 1.5% agar-agar to potato dextrose broth and then sterilized by autoclaving.

2.4 Inoculation and incubation

The samples (water suspensions) were first inoculated on to the PDA and incubated at 30°C for 72-96 hours and growth pattern was studied according to the suggestions and methods of Harrigan (1998). The selected colonies from PDA were further transferred to PDB for growth of cells.

2.5Identification of as per gillusniger

Aspergillus niger was identified on the basis of morphology and growth pattern according to the methods recommend by Harrigan (1998) [13]. It was based on general examination of growth pattern of mycelia and spores under microscope after staining.

2.6 Ultrasonic measurement studies

Firstly, the 10 mL potato dextrose broth (without inoculum) was poured into the sonication vessel (control)(15 ml glass bottles, internal diameter 21mm, flat base, 2.5 mm wall thickness). Next, the low power (2 mW) ultrasonic transducer of 10mm gap between transmitting and reflecting surfaces was immersed into the vessel. The other end of the transducer is connected to the ULTRASONIX 4400M (fabricated and supplied by Roop Telsonic Ultrasonix Limited, Mumbai.) by BNC cable and Pulse Echo pattern was observed on the CRO screen which was used to measure delay time and attenuation. Further the selected cells of A.niger from PDA inoculated into the 10 mL PDB which were poured into the sonication vessels with cell density ranging from 10^1 to 10^8 cells/ml insteps of 10^1 increments. For each cell density the ultrasonic velocity and attenuation coefficient measurements were repeated for six times and average values were reported in tables. The sonication vessel kept inside the water bath upto its neck and the temperature of the water bath was maintained at constant temperature of 303K with aid of automatic temperature control unit.

2.7 Ultrasonic disruptive studies

For the ultrasound irradiation purpose the control samples (without sonication) of A.niger cultures were selected with 10^7 cell/ml. PDB inoculated 10^7 cell/ml took into the different number of sonication vessels (15 ml glass bottles, internal diameter 21mm, flat base, 2.5 mm wall thickness to a depth of 25 mm) of 10 ml each exposed to ultrasound for a period of 1 min to maximum of 6 min at 2 MHz and 1 min to maximum of 8 min at 10 MHz by using ultrasonic-processor (model USG – 600) series and an output power of 600 W i.e. 60 W/ml. Throughout the study a sonotrode of 12 mm diameter of titanium probe set at 5 mm below the surface of

the culture was used. After sonication, the densities of viable cells were counted by using hemocytometer. The measurements were repeated at each sonication minute for six times and average values were reported in the tables.

2.8 Ultrasonic velocity measurements

The novel Pulse Echo Overlap (PEO) technique was introduced to measure ultrasonic parameters in PDB of A.niger. The ultrasonic transducer generates a pulse of ultrasound which travels across the sample, was reflected from the back wall of the measurement cell, travels back through the sample, and was then detected by the same transducer. The ultrasound velocity in the medium was found from the measured delay time difference (Δt) and earlier found length of the measurement chamber (d).

(1) $$v = \frac{2d}{\Delta t}\ cm/sec$$

The uncertainty in velocity measurements by this technique were ± 0.02%.

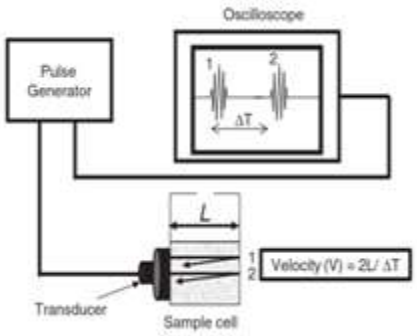

Figure 1: Schematic representation of Ultrasonic velocity measurements

2.9 Attenuation Coefficient Measurements

The attenuation coefficients of the bacteria cultures were carried out by measuring the amplitudes of transmitted pulses of selected two successive echoes on CRO screen. The ultrasonic attenuation coefficient was calculated by using the following formula. $\alpha = \frac{1}{2l}\ln\left(\frac{A_n}{A_{n+1}}\right)$ nep/cm (2)

Where $2l$ was distance travelled and A_n / A_{n+1} was the ratio between two successive echoes of A_n and A_{n+1}. The uncertainty attenuation coefficient **(α)** measurements were ±0.0015%.

2.10 Measurement of Cell Density

The hemocytometer was a device used to count cells. It consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber was engraved with a laser-etched grid of perpendicular lines. The device was carefully crafted so that the area bounded by the lines was known, and the depth of the chamber was also known. It was therefore possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.

2.11 Density measurements

The density of bacteria cultures were carried out by using bicapillary pyknometer of 10 ml volume. The transfer of A.niger cultures was made by using micro pipette in the laminar flow chamber to avoid contamination with air and body.The density of bacteria cultures was measured by using the following procedure;

Mass of the empty bicapillary pyknometer = $w_1$gm
Mass of the bacteria culture + pyknometer = $w_2$gm
Mass of the culture (m) = $w_2 - w_1$gm
Volume of the culture = V cm^3
Density of the bacteria culture (ρ) =

$$\frac{Mass\ \ of\ \ the\ \ culture\ (m)}{Volume\ \ of\ the\ \ culture\ (V)}\ \ gm/cm^3\ (4)$$

The accuracy in measuring density of bacteria cultures was 2 parts in 10^5. A single pan electrical balance was used to find the masses of the samples to find out density. The accuracy of measuring the masses was ±0.01mg**.**

2.12 Computed parameters

2.12.1Compressibility (β_s)

The compressibility can be calculated as

$\beta_s = 1/\rho v^2\ cm^2/dynes$ (5)

Where ρ is the density and ν is the ultrasonic velocity

2.12.2 Free Length (L_f)

The equation to calculate free length is
$L_f = K\sqrt{\beta_s}$ A.U. (6)
Where K is Jacobson's temperature constant was = 631 x 10^{-6} at 303 K

2.12.3 Acoustical Impedance (z)

The equation to calculate acoustical impedance is
Z = ρν (7)
Where ρ was the density and ν was the ultrasonic velocity

3. Result and Discussions

The measured and evaluated ultrasonic parameters as function of sonication time in PDB cultures with varied cell density for A.niger and P.chrysogenum were reported in Table (1) and Table (2) at 2 and 10 MHz ultrasound. The compared ultrasonic parameters at 2 and 10 MHz were depicted from figure (2) to figure (8). The control samples were chosen with cell density of 10^7cells/ml. It was noticed that P.chrysogenum cultures possessed high density than A.niger. It was found that density of PDB cultures decreased with increased sonication time as shown in figure (2).

The ultrasonic velocities of A.niger and P.chrysogenum varied as a function of sonication time at 2 and 10 MHz as shown in figure (3). Cell sizes of A.niger and P.chrysogenum were cited in literatures as falling in the ranges from 25 to 50 µm (Raper et al; 1965) and 15 to 30 µm (Raminez; 1982)

respectively. It was generally admitted that cells of a bigger size were more sensitive to ultrasound. Thus A.niger cells were more detrimental to ultrasound than P.chrysogenum cells. It was observed that unlike in bacteria cultures the variation of ultrasonic velocities in fungus cultures were different with sonication time. The primary reason could be their bigger cell sizes. Due to this they did not undergo declumping process instead transient cavitation directly initiated which was held responsible for cell disruption. It was observed that ultrasonic velocity linearly decreased with sonication time at 2 and 10 MHz as shown in figure (3). It was found that the ultrasonic velocity of A.niger varied relatively faster than P.chrysogenum with sonication time. It was investigated that the time taken at 10 MHz was more compared to 2 MHz to decrease ultrasonic velocity to minimum. It was because the formation of transient cavitationswas more destructive at 2MHz than 10 MHz. Further, it was found that compressibility and free length linearly increased because concentration of cells present in PDB cultures decreased with sonication time. It was highlighted that the increased compressibility confirmed the decreased ultrasonic velocity with sonication time. The parallel measured viable cells per ml (figure (8)) further ascertained thatthe decreased ultrasonic velocity with sonication time.

It was observed that attenuation coefficient decreased with sonication time for A.niger and P.chrysogenum as shown in figure (6). Figure (8) showed that cell concentration steeply decreased with sonication time. It indicated that the numbers of scatters present in PDB were decreased with sonication time. As a result scattering losses drastically fell down with sonication time that contributed to overall decrease of attenuation coefficient. From the varied ultrasonic coefficient, it was predicted that A.niger strongly interacted at 2 MHz but P.chrysogenum strongly interacted at 10 MHz because of their respective cell sizes. The decreased acoustic impedance was in good agreement with decreased attenuation coefficient.

4. Tables

Table 1: Sonication time (t), Density (ρ), Ultrasonic velocity (V), adiabatic compressibility (β_s), free length (L_f), Attenuation coefficient (α), Acoustical Impedance (z) and Number of viable cells per ml of A.niger

t min.	*ρ(gm/cm³)*	*V×10² cm/sec*	*β_s× 10⁻¹¹ cm²/dyn*	*L_f× 10⁻⁹ cm*	*αnep/cm*	*z ×10⁵ gm/cm²-sec*	*Viable cells/ml*
0	1.015	1595	3.873	3.926	0.4301 /0.76/	1.618	10^7
2MHz							
1	1.015	1590	3.897	3.939	0.4412	1.613	$5x10^6$
2	1.015	1580	3.946	3.963	0.4400	1.603	$6x10^5$
3	1.013	1565	4.03	4.005	0.4302	1.585	10^4
4	1.011	1540	4.171	4.075	0.3100	1.556	400
5	1.011	1530	4.225	4.101	0.2956	1.546	200
6	1.011	1525	4.253	4.115	0.2898	1.541	40
10	MHz						
1	1.015	1593	3.882	3.931	0.75	1.616	10^7
2	1.014	1587	3.915	3.948	0.7485	1.609	$6x10^6$
3	1.013	1575	3.979	3.981	0.7015	1.595	$9x10^5$
4	1.011	1560	4.064	4.022	0.6615	1.577	$8x10^4$
5	1.011	1555	4.09	4.035	0.605	1.572	1000
6	1.011	1536	4.192	4.085	0.5341	1.552	300
7	1.011	1527	4.242	4.109	0.5187	1.543	150
8	1.010	1522	4.274	4.125	0.5019	1.537	15

Table 2: Sonication time (t), Density (ρ), Ultrasonic velocity (V), adiabatic compressibility (β_s), free length (L_f), Attenuation coefficient (α), Acoustical Impedance (z) and Number of viable cells per ml of P.chrysogenum.

t min.	*ρ(gm/cm³)*	*V×10² cm/sec*	*β_s× 10⁻¹¹ cm²/dyn*	*L_f× 10⁻⁹ cm*	*αnep/cm*	*z ×10⁵ gm/cm²-sec*	*Viable cells/ml*
0	1.136	1613	3.383	3.67	0.3642 /0.861	1.832	10^7
2MHz							
1	1.135	1610	3.399	3.678	0.3621	1.827	$6x10^6$
2	1.130	1600	3.456	3.709	0.3568	1.808	10^5
3	1.127	1582	3.545	3.756	0.3439	1.782	10^4
4	1.12	1570	3.622	3.797	0.3400	1.758	10^3
5	1.11	1542	3.788	3.883	0.2414	1.711	400
6	1.09	1530	3.919	3.95	0.20	1.667	120
7	1.08	1524	3.986	3.983	0.197	1.645	45
8	1.08	1519	4.013	3.997	0.197	1.640	20
10	M HZ						
1	1.136	1612	3.387	3.672	0.8610	1.831	10^7
2	1.135	1604	3.424	3.692	0.8599	1.820	$6x10^6$
3	1.134	1595	3.466	3.714	0.852	1.808	$3x10^5$
4	1.132	1584	3.521	3.744	0.845	1.793	$9x10^4$
5	1.13	1570	3.591	3.781	0.710	1.774	$5x10^4$
6	1.124	1551	3.698	3.837	0.592	1.743	10^4
7	1.11	1538	3.808	3.893	0.425	1.707	$2x10^3$
8	1.09	1530	3.919	3.95	0.40	1.667	300
9	1.07	1525	4.018	3.999	0.39	1.631	90
10	1.07	1520	4.045	4.013	0.39	1.626	22

5. Graphs

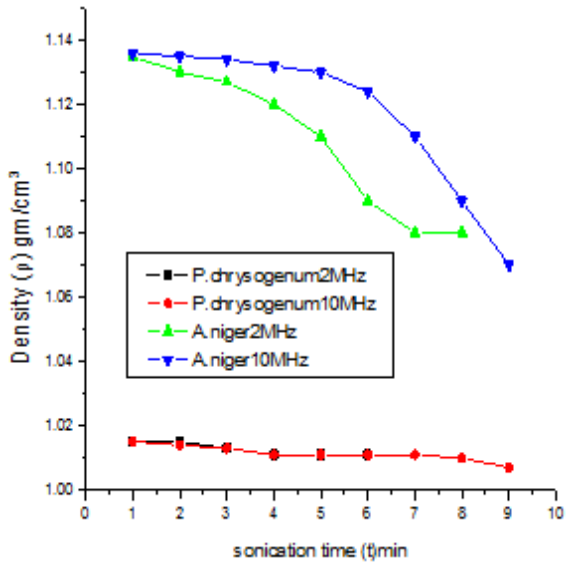

Figure 2: Comparison of density as function of sonication time at 2 & 10 MHz

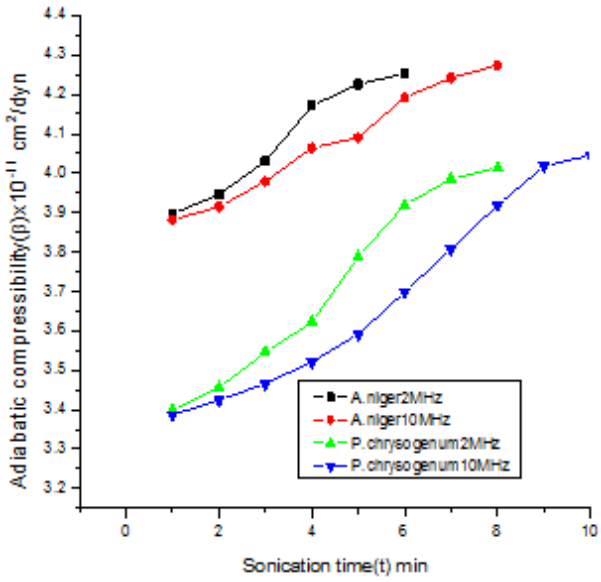

Figure 4: Comparison of compressibility as a function of sonication time at 2 & 10 MHz

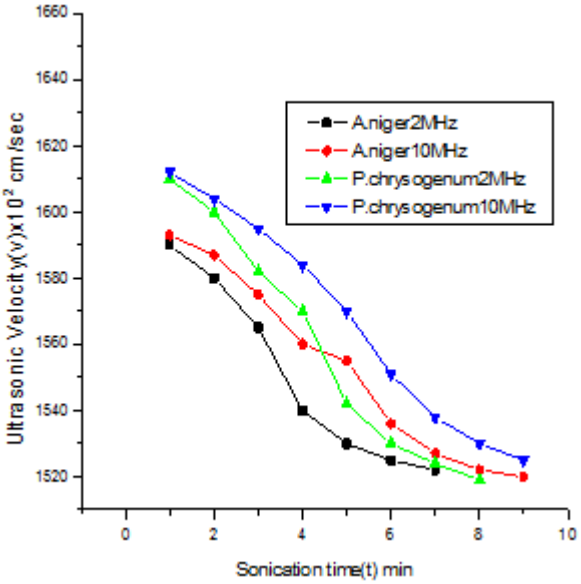

Figure 3: Comparison of ultrasonic velocity as a function of sonication time at 2 & 10 MHz

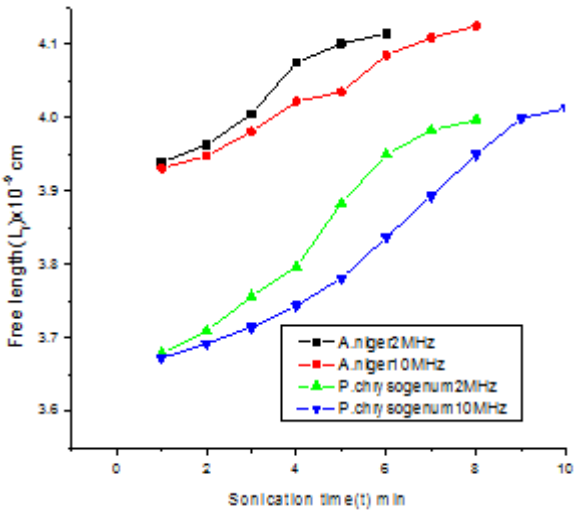

Figure 5: Comparison of free length as a function of sonication time at 2MHz & 10MHz

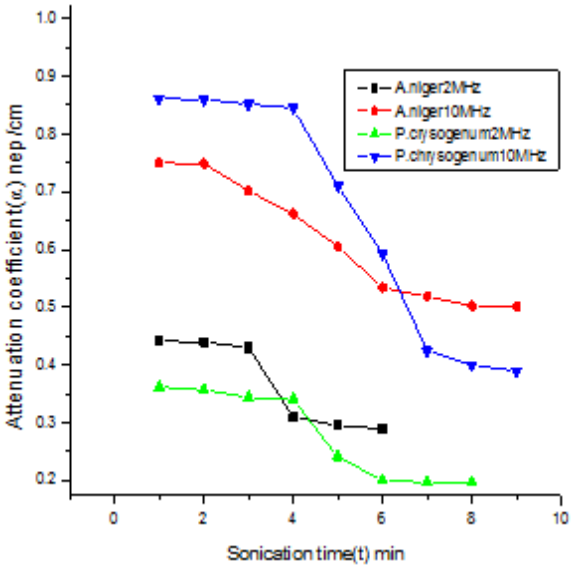

Figure 6: Comparison of attenuation coefficient as a function of sonication time at 2 & 10 MHz

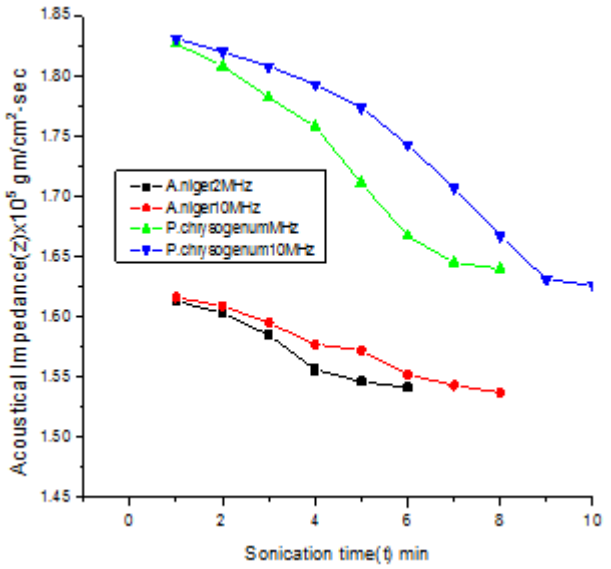

Figure 7: Comparison of acoustical impedance as a function of sonication time at 2 & 10 MHz

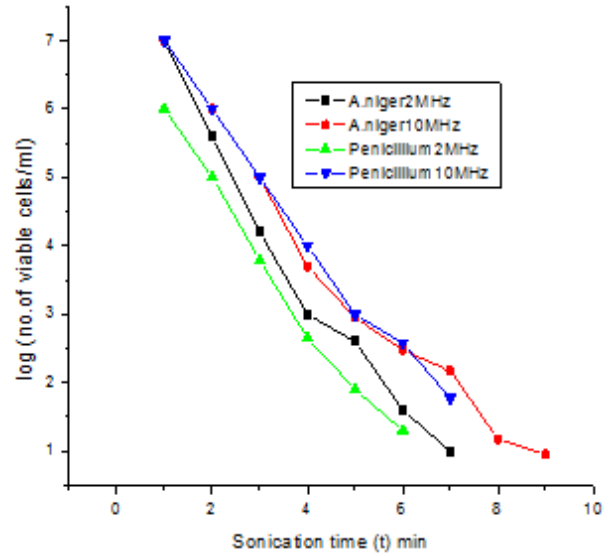

Figure 8: Comparison of number of viable cells per ml as a function of sonication time at 2 & 10 MHz

6. Conclusions

It was concluded that the ultrasonic velocity and attenuation coefficient strongly depends on cell size and number of cell present in PDB. It was hypothesized that scattering losses were occurred more at 2 MHz for A.niger and for P.chrysogenum at 10 MHz. It was learnt that the data of ultrasonic parameters could be used to estimate the fungal cell density. It was also learnt that the variation of ultrasonic parameters with sonication time could be used for image production.

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